U.S. Army Center for Health Promotion and Preventive Medicine

USACHPPM

TOXICOLOGY STUDY NO. 87-XE-0BT9-09
PROTOCOL NO. 0BT9-60-09-05-01
MECHANISM OF RDX-INDUCED SEIZURES IN RATS
SEPTEMBER 2009

Preventive Medicine Survey: 40-5f1

Approved for public release; distribution unlimited.

Readiness Thru Health

REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
09-09-2009	Environmental Health Assesment	Feb. - 2009 - Sept. - 2009

4. TITLE AND SUBTITLE
Toxicology Study No. 87-XE-0BT9-09, Mechanism of RDX-Induced Seizures in Rats

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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Aberdeen Proving Ground, MD 21010-5403

8. PERFORMING ORGANIZATION REPORT NUMBER
Toxicology Study No. 87-XE-0BT9-09

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Army Environmental Center IMAE-CD, US Army Environmental Command, 5179 Hoadley Road, Aberdeen Proving Ground, MD 21010-5401

10. SPONSOR/MONITOR'S ACRONYM(S)
AEC

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Unlimited; approved for public release

13. SUPPLEMENTARY NOTES

14. ABSTRACT
RDX is found in soil and ground water in and surrounding training ranges, creating potential hazards to human health. Oral RDX over-exposure causes seizure in rats and humans, the mechanism of which is unknown. In this study rats were dosed orally at 75 mg/kg RDX to induce seizure. The brain concentration of RDX was determined in samples taken from rats euthanized at the time of seizure onset; brain acetylcholinesterase was also measured. Also, RDX was screened for affinity to a library of brain receptors to determine if RDX affected any seizure-related targets. Brain concentrations of RDX were greater than 8 µg/g wet wt. in the animals that seized. RDX was found to bind exclusively to the convulsant site on the GABAa receptor with an IC50 of 22 µM. The mechanism of RDX-induced seizure is likely due to dis-inhibition of excitatory neurons by blockage of the GABA-mediated inhibitory chloride current. This valuable information contributes mode of action insights that can be used in the physiologically-based pharmacokinetic modeling to extrapolate rat data to human.

15. SUBJECT TERMS
gamma-amino butyric acid (GABA), RDX, acetylcholinesterase, convulsions, seizure, rat

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
UU	UU	UU	UU		Larry Williams, Ph.D.
					19b. TELEPHONE NUMBER *(Include area code)* 41-436-7159

Reset

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Study Title

MECHANISM OF RDX-INDUCED SEIZURES IN RATS

TOXICOLOGY STUDY NO. 87-XE-0BT9-09

Data Requirement

Not Applicable

Authors

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Study Completed

Final Report

Performing Laboratory

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STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

1. NO CLAIM OF CONFIDENTIALITY UNDER FIFRA (OR CHANGE OTHER APPROPRIATE GLP REGULATION).

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA (or change to other appropriate GLP regulation) section 10(d)(1)(A), (B), or (C).

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SAMPLE GOOD LABORATORY PRACTICE STATEMENTS

This study meets the requirements for 40 CFR Part 160 (or change to appropriate GLP regulations). Submitter is defined as a representative of the organization submitting study to EPA or FDA.

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EXECUTIVE SUMMARY
TOXICOLOGY STUDY NO. 87-XE-0BT9-09
MECHANISMS OF RDX-INDUCED SEIZURES IN RATS

1. PURPOSE. The purpose of this study is to determine the role brain acetylcholinesterase (AChE) enzyme plays in the mechanism(s) of RDX-induced seizure and to determine the brain concentration of RDX at the time of seizure onset. In addition, RDX was screened for affinity to a library of brain neurotransmitter receptors to determine if the mechanism of seizure involved; activation or inhibition of specific seizure-related targets. This valuable information will be used in conjunction with the physiologically-based pharmacokinetic modeling to refine Reference Dose through a more accurate extrapolation of rat data to humans. This work will provide mode of action information regarding the acute effect of RDX on humans and possibly wildlife, ultimately helping to determine less uncertain environmental exposure levels while maintaining public health.

2. CONCLUSIONS.

a. RDX has significant affinity for the convulsant of the gamma-amino butyric acid ($GABA_A$) receptor. Thus, the mechanism of RDX-induced seizure is very likely to be dis-inhibition of excitatory neurons due to blockage of the inhibitory Cl^- current mediated by GABA (Bigler, 1977; Ramakrishnan et al., 2005).

b. Acetylcholinesterase (AChE) inhibition is not involved in the mechanism of seizure induction.

c. The concentration of RDX in brain must reach a level greater or equal to 8 μg/g wet weight before seizure can begin. Higher brain concentrations can shorten the time to seizure onset.

d. These data will provide valuable information to the physiologically-based pharmacokinetic modeling being used to extrapolate rat data to human (Krishnan et al., 2009) and assist in reducing the interspecies uncertainty associated with extrapolating safe levels in animals with those for humans.

3. RECOMMENDATIONS: Apply the information from this report to the reassessment of interspecies uncertainty associated with extrapolating safe levels in animals with those for humans.

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TOXICOLOGY STUDY NO. 87-XE-0BT9-09
MECHANISMS OF RDX-INDUCED SEIZURES IN RATS

1. REFERENCES. See Appendix A for a listing of references.

2. AUTHORITY. Pursuant to Army Regulation (AR) 40-5(2007) and AR 70-1(2003) and to ensure environmental safety and occupational health (ESOH) as part of the responsibilities outlined in AR 200-1(2007). This study was sponsored by Army Environmental Command (AEC), Installation Restoration Program and was completed to understand the pharmacokinetics and toxicodynamics to reduce the uncertainty associated with the reevaluation of the RDX risk assessment and refinement of the prediction of adverse health effects from environmental exposures.

3. PURPOSE. The purpose of this study was to determine the role brain acetylcholinesterase (AChE) enzyme plays in the mechanism(s) of RDX-induced seizure and to determine the brain concentration of RDX at the time of seizure onset. In addition, RDX was screened for affinity to a library of brain neurotransmitter receptors to determine if the mechanism of seizure involved activation or inhibition of specific seizure-related targets. This valuable information will contribute to the physiologically-based pharmacokinetic modeling being used to extrapolate rat data to human. This work will provide mode of action information regarding the acute effect of RDX on humans and possibly wildlife, ultimately helping to determine more accurate environmental media levels that ensure human health and the environment.

4. GENERAL BACKGROUND.

a. RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine, hexogen, Royal Demolition eXplosive) is an explosive widely used by the military since WWII and has been found in soil and ground water in and surrounding training ranges, creating potential hazards to the environment and human health (ATSDR, 1995). Humans can be exposed during manufacture, use, or when unexploded ordinance (UXO) lies on soil at Army training sites (ATSDR, 1995). RDX is present in the environment surrounding training ranges and its use at training ranges continues. The currently used benchmarks to safeguard human health are under evaluation by the US Environmental Protection Agency (USEPA) in the reevaluation of cancer slope factors, reference dose (RfD) determination and subsequent drinking water Health Advisories. Due to this contamination, primarily in drinking water, human exposure is possible. Clean-up goals for sites contaminated with soil-borne metals and organic compounds often are established on the basis of risk assessments that use regulatory standards which are in turn based largely upon a combination of animal studies and numeric default factors that account for uncertainty in the data.

b. Although the USEPA has defined RDX as a possible human carcinogen based on an early report of hepatocellular carcinomas in female (not male) B6C3F1 mice (Lish et al., 1984), the work was circumstantial and is being reassessed (Parker et al., 2006). RDX is not mutagenic in the standard genotox assays and is not teratogenic in rats or rabbits (Cholakis et al., 1980; Reddy et al., 2005). For non-carcinogenic effects, the oral RfD for RDX of 3 μg/kg/day is based on increased incidence of prostatic inflammation in a 24 month rat study (Levine et al., 1983). However, the Longer-Term Health Advisory (LTHA) for RDX (0.1 mg/L in children and 0.35 mg/L in adult) is based on the No-Observed-Adverse-Effect-Level (NOAEL) in seizing monkeys (Martin et al., 1974). The USEPA acute oral minimum risk level (MRL) of 0.06 mg/kg/day is based on the 20 mg/kg/day dose of RDX-induced convulsions in animals (Angerhofer et al., 1986). Uncertainty factors used in these calculations include a denominator of 100 to adjust for uncertainty in the NOAEL.

c. Currently, the USEPA is in the process of re-evaluating the RfD for RDX that is used to determine clean-up goals for contaminated sites. Knowledge of the precise mechanism of action of RDX seizure induction will reduce the uncertainty factors associated with the risk assessment and result in a less uncertain, more scientifically-defensible safe benchmark for RDX.

d. The main acute effect of RDX after ingestion of high doses is the induction of seizures accompanied by excessive salivation (Barsotti et al., 1949; Kaplan et al., 1965; Katel et al., 1972; Kucukardali et al., 2003). RDX-induced seizures also occur in experimental animals and most animals that seize demonstrate excessive salivation (Schneider et al., 1977; Burdette et al., 1988; Crouse et al., 2006). These clinical signs are similar to the clinical signs of organophosphate pesticides and nerve agents (McDonough et al., 1997). The mechanism of organophosphate seizure induction is known to be initiated by a 70% inhibition of brain AChE leading to subsequent potentiation of brain muscarinic receptor activation (McDonough et al., 1997; Barthold et al., 2005).

e. RDX is reported to inhibit brain AChE up to 25% at 6 hr after a low dose (6-12 mg/kg) i.p. administration; a dose to rats that did not induce seizures (Brown, 1975). However, AChE was not evaluated at doses of RDX that induced seizure at the time of seizure. Thus, the true extent of brain AChE inhibition in seizing rats is not known, and it is uncertain if the level of AChE inhibition would be sufficient to be responsible for seizure induction by itself, i.e., the 70% necessary for nerve agents (McDonough et al., 1997). However, RDX may have other mechanisms for seizure induction. Results from a very recent rat brain ligand-binding study suggest that RDX binds to the convulsant site of the $GABA_A$ receptor with an inhibitory dissociation constant (K_i) of 22 μM. Blockage of chloride flux at the $GABA_A$ receptor with convulsants such as picrotoxin is known to induce seizure (Bigler, 1977; Ramakrishnan et al., 2005). If brain RDX concentrations reached 22 μM following an overdose, this may be the mechanism of RDX-induced seizure. However, seizure induction may occur from a combination of RDX effects on brain chemistry including an inhibition of brain AChE. To understand the role of AChE in RDX-induced seizure, we need to determine the level of AChE inhibition at the onset of seizure and whether it achieves a level of inhibition necessary for nerve agent seizure induction, i.e., 70% of control. In addition, knowing the correlation of the plasma and brain concentration of RDX at the time of seizure onset will provide valuable information to the physiologically-based pharmacokinetic modeling being used to extrapolate rat data to human (Krishnan et al., 2009). This work will provide information regarding the acute effect of RDX on humans and possibly wildlife, ultimately helping to set realistic environmental levels.

f. The following table identifies the critical dates of the acute and 14-day studies.

Critical Event	Date of Event
Animal Use Protocol Approved	5/21/09
Animals Received	7/1/09
Study Start	7/5/09
Experimental Start	7/5/09
Post seizure Necropsies	7/5/09 and 7/6/09
Experimental Completion	7/6/09
Study Completion	9/16/09

5. MATERIALS.

a. Test Article. Hexahydro-1,3,5-Trinitro-1,3,5-Triazine, RDX, was produced at Holston Army Ammunition Plant and analyzed by HPLC and determined to be >99.5% pure. The dosing solution was prepared by suspension of RDX at 37.5 mg/ml in 1% methylcellulose, 0.1% Tween 80 in distilled water (vehicle). Control animals were dosed with the vehicle exclusively. Dosing solution was verified by Directorate of Laboratory Sciences (DLS) at 34.8 mg/ml using GC-ECD (SOP CAD 86.2 and SOP CAD 108.1) (Bishop et al., 2003).

b. Reagents – All reagents were obtained from Aldrich-SIGMA, St. Louis, MO

- Sodium Phosphate Dibasic (Na_2HPO_4).
- Sodium Phosphate Monobasic (NaH_2PO_4).
- Triton X-100
- 5,5'-dithio-bis[2-nitrobenzoic acid] (DTNB)
- Sodium bicarbonate ($NaHCO_3$)
- Acetylthiocholine iodide

c. Materials.

- Cervical Dislocator, Otto Environmental LLC, Milwaukee, WI
- Polytron AJ 10/35, Brinkman Instruments, Rexdale, Ontario, Canada
- Bio-Tek Synergy HT, Winoosk, VT
- Agilent 6890 Gas Chromatograph with Electron Capture Detection (GC-ECD)

6. METHODS.

a. Experimental Approach.

(1) The study described was conducted in a manner consistent with the principles of the Good Laboratory Practice (GLP) regulations in the Toxic Substances Control Act (TSCA): 40 CFR (Code of Federal Regulations) 792, plus amendments (Title 40).

(2) Dosing of animals with RDX was patterned after the observations of Way and McCain, 2007. They dosed rats with 75 mg/kg (15 mg/ml) in 1% methylcelluose/0.2% Tween 80; this dose induced seizure in all animals with an average time to seizure onset of 11 min. For the present mechanistic study, animals were pair-dosed by oral gavage with either vehicle (1% methylcelluose/0.2% Tween 80, 2 ml/kg, n=8) or RDX at 75 mg/kg (37.5 mg/ml, 2 ml/kg) in 1% methylcelluose/0.2% Tween 80, n=8. All oral dosing was administered using an oral gavage needle (16 - 18 ga. x 2 inch) and a 1 ml syringe. Rats were fasted overnight (not to exceed 18 hrs while maintaining free access to water) prior to dosing to maximize and normalize absorption of RDX (Way et al., 2007).

(3) Animals were observed continuously for 30 min post-dosing for respiratory distress and seizure. Respiratory distress due to mal-administration of the oral gavage needle was not observed in this experiment.

(4) The process of RDX-induced seizure includes a gradient of seizure signs with increasing severity: behavioral changes (increased agitation), fasciculation (twitching), tremors, brief seizures (few seconds), visible convulsions, full seizure (tonic-clonic), and coma (Way et al., 2007). After RDX dosing, when the rat progressed from tremor to a seizure of at least 2 second duration (as defined by Way and McCain as a brief seizure), the animal was euthanized via cervical dislocation using the Otto Cervical Dislocator. If the RDX-dosed animal did not seize within 30 min., the animal was euthanized as mandated by the CHPPM Institutional Animal Care and Use Committee (IACUC), i.e., no animal survived longer than 30 min. post dosing.

(5) Following cervical dislocation, the thorax was opened and a 100 µl heart blood sample was taken via intracardiac puncture (TOX SOP 053), and immediately diluted in a 100 ml volumetric flask for subsequent DLS analysis. The brain was then removed and dissections taken for chemical analysis. Specifically, a coronal razor cut was made at the level of the optic chiasm with the rostral portion including the bulk of the frontal cortex. The hemispheres were separated, and individually weighed; the left frontal cortex was frozen on dry ice and stored in a -80°Cfreezer until later analysis for AChE activity, and the right frontal cortex was maintained on ice (~4°C) until extraction that same day by DLS. The paired, vehicle-treated, control animal was euthanized immediately following the RDX-treated animal.

(6) Blood and brain samples were given to the USACHPPM DLS for determination of explosive concentration in blood using GC-ECD (SOP CAD 86.2 and SOP CAD 108.1) (Bishop et al., 2003).

(7) In a separate study, RDX in dimethylsulfoxide (DMSO) solution was provided to MDS Pharma, Inc., Taipei, Taiwan, for screening in their library of central nervous system receptor binding assays (catalog numbers are provided in Table 4). The first screening was done against 25 different receptors using RDX at a concentration of 33 μM. When affinity for the $GABA_A$ was discovered, a comprehensive concentration curve was generated to calculate the 50% inhibitory concentration (IC_{50})and K_i.

b. Procedures.

(1) AChE activity in frontal cortex homogenate was determined using the 96-well microplate adaptation of the Ellman method (Padilla et al., 1998). A 2% (wet wt./vol.) homogenate was made in 0.1 M Na phosphate buffer (pH 8.0) + 1% Triton, using a 20 sec burst (on ice) of a Polytron. Total cholinesterase (brain is 95% AChE) was determined at 37°C by measuring the change in absorbance at 412 nm over 5 min in a 96 well plate reader using acetylcholine iodide as substrate DTNB as the colorimetric indicator. A glutathione sulfhydryl standard curve was generated for conversion of absorbance units into nmol of sulphydryl groups. AChE enzymatic activity in the brain samples was then calculated as μmol substrate hydrolyzed/min/g wet wt. (Padilla et al., 1998). Statistical significance was determined using Students t-Test with $\rho \leq 0.05$.

(2) RDX concentration in blood and brain samples was determined using an Agilent 6890 GC-ECD according to SOP CAD 86.2 and SOP CAD 108.1. Briefly, the blood dilution and brain homogenates were extracted into isoamyl acetate and an appropriate volume run against RDX standards in the GC-ECD.

7. RESULTS.

a. Time to RDX-Induced Seizure. The first two rats dosed with RDX did not seize within 30 min. and were immediately euthanized along with their vehicle-treated pair. Six of the next eight RDX-treated animals did seize within 30 min.; a total of only six vehicle-treated animals were collected for subsequent analysis. Pertinent animal data including time to seizure is presented in Table 1.

Table 1. Time to RDX-Induced Seizure

Rat #	Body Wt. (g)	Treatment	Time to Seizure (min.)
R09-0778	160	Veh	dns
R09-0779	155	Veh	dns
R09-0780	158	RDX	>30
R09-0781	172	RDX	>30
R09-0782	159	RDX	18
R09-0783	165	RDX	16
R09-0784	169	RDX	10
R09-0785	160	Veh	dns
R09-0786	169	RDX	21
R09-0787	164	Veh	dns
R09-0788	170	RDX	14
R09-0789	159	Veh	dns
R09-0790	161	RDX	>30
R09-0791	163	Veh	dns
R09-0792	172	RDX	17
R09-0793	169	RDX	>30

dns = did not seize

b. Brain AChE Activity. There was no effect of RDX treatment on brain total cholinesterase activity; the average activity in each group was 6.6 µmol/min/g wet wt. The results are presented in Table 2.

Table 2. Brain AChE Activity

Rat #	Treatment	AChE Activity µmol/min/g		Rat #	Treatment	AChE Activity µmol/min/g
R09-0778	Veh	6.6		R09-0780	RDX	7.0
R09-0779	Veh	6.0		R09-0781	RDX	6.5
R09-0785	Veh	6.9		R09-0782	RDX	6.7
R09-0787	Veh	6.3		R09-0783	RDX	7.4
R09-0789	Veh	7.2		R09-0784	RDX	6.5
R09-0791	Veh	6.3		R09-0786	RDX	6.4
				R09-0788	RDX	6.9
	Avg	*6.6*		*R09-0790*	*RDX*	*6.8*
	Stdv	0.5		R09-0792	RDX	6.1
				R09-0793	RDX	6.1
					Avg	6.6
					Stdv	0.4

c. RDX Concentration in Blood and Brain

(1) RDX in the blood and brains of vehicle-treated animals was below the level of detection. The measured concentration of RDX in blood and brain samples is presented in Table 3; time to seizure is also shown. The correlations of blood concentration to brain concentration are illustrated in Figure 1; data for Rat# R09-0780 was removed as an outlier as the blood concentration was greater than 2 s.d. from the mean. There was a positive correlation with a coefficient of 0.569.

(2) The correlations of blood and brain RDX concentration with time to seizure are presented in Figures 2A and 2B. There was a positive correlation of brain RDX concentration with time to seizure with a coefficient of 0.606.

Table 3. RDX Concentration in Blood and Brain

Rat #	Blood Conc. (μmol/ml)	Brain Conc. (μmol/g wet et.)	Time to Seizure (min.)
R09-0780	11.9	12.4	dns
R09-0781	5.8	10.8	dns
R09-0782	5.5	9.9	18
R09-0783	3.8	8.7	16
R09-0784	4.3	11.1	10
R09-0786	3.5	8.1	21
R09-0788	4.7	10.0	14
R09-0790	3.8	7.3	dns
R09-0792	4.6	11.5	17
R09-0793	5.2	9.1	dns
Avg	5.3	9.9	16
Stdv	2.4	1.6	3.7

dns – Did Not Seize

Figure 1. **A** - Correlation of Blood RDX concentration and Brain RDX Concentration at Time of Seizure – Line NOT forced through zero
B - Correlation of Blood RDX concentration and Brain RDX Concentration at Time of Seizure – Line forced through zero

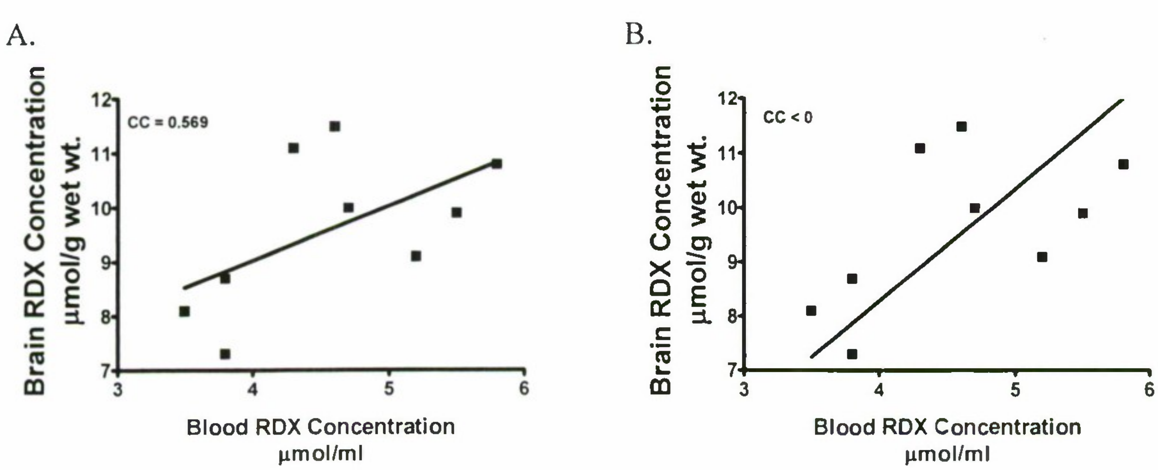
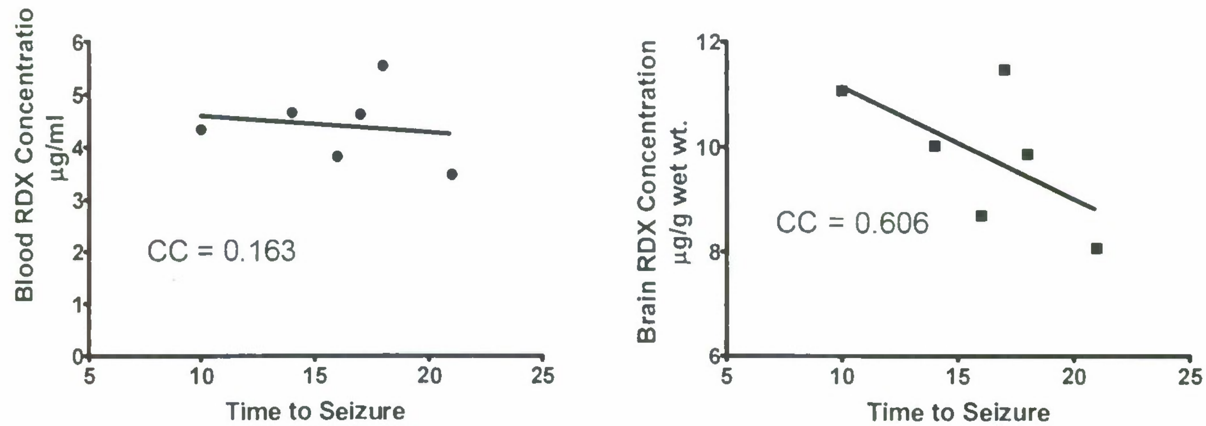


Figure 2. A - Correlation of Blood RDX Concentration to Time of Seizure.
B - Correlation of Brain RDX Concentration to Time of Seizure.

A.

B.

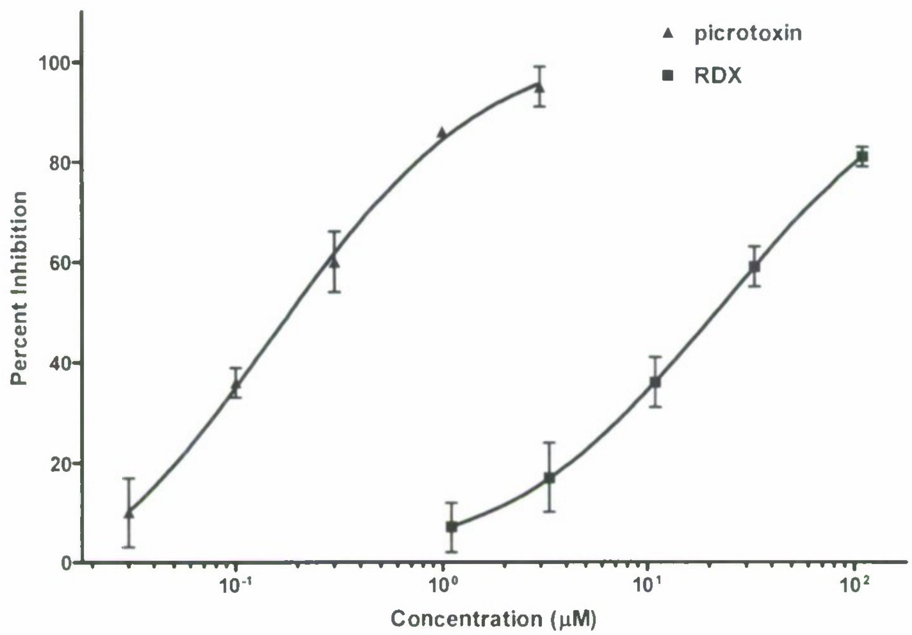


d. RDX Affinity for the $GABA_A$ Receptor - The receptors assayed included those implicated as targets of known convulsants such as the glutamate family of receptors, nicotinic and muscarinic acetylcholine receptors, the glycine receptor, the site 2 sodium channel, and the family of $GABA_A$ ligand sites, as well as several others. A complete list is provided in Table 4. Out of this comprehensive list, RDX had affinity only for the convulsant site on the $GABA_A$ receptor, displacing the test ligand, [^{35}S]-t-butylbicyclophospho-orothionate (TBPS) (Maksay, 1993) , with an IC_{50}/K_i of 22 µM. The full dose response curve is shown in Figure 3; RDX has an apparent K_i of 22 µM compared to picrotoxin with an apparent K_i of 0.2 µM. RDX has a potency similar to pentylenetetrazol a convulsant commonly used in animal models of seizure and epilepsy (Coulter et al., 1990).

Table 4 – List of Binding Assays Screened for Affinity of RDX

MDS PHARMA CATALOG NUMBER	RECEPTOR/BIDING SITE	% INHIBITION
235010	GLUTAMATE, NON-SPECIFIC	7
232600	GLUTAMATE, AMPA	-10
233000	GLUTAMATE, NMDA, PHENCYCLIDINE	2
258590	NICOTINIC ACETYLCHOLINE	9
254000	MUSCARINIC, NON-SELECTIVE, CENTRAL	7
239000	GLYCINE, STRYCHNINE-SENSITIVE	2
279510	SODIUM CHANNEL, SITE 2	16
228510	GABA, NON-SELECTIVE	4
226810	$GABA_A$, CHLORIDE CHANNEL, TBOB	76*
226830	$GABA_A$, CHLORIDE CHANNEL, TBPS	78*
226600	$GABA_A$, FLUNITRAZEPAM, CENTRAL	5
226500	$GABA_A$, MUSCIMOL, CENTRAL	13
203500	ADRENERGIC A_1, NON-SELECTIVE	-11
203900	ADRENERGIC A_2, NON-SELECTIVE	-5
260500	OPIATE, NON-SELECTIVE	7
226700	PERIPHERAL BENZODIAZEPINE RECEPTOR	-6
268700	PURINERGIC P_{2X}	10
271000	SEROTONIN 5-HT_1,NON-SELECTIVE	26
271200	SEROTONIN 5-HT_{1B}	-5
271910	SEROTONIN 5-HT_3	-9
272000	SEROTONIN 5-HT_4	12
279510	SODIUM CHANNEL, SITE 2	-5
220320	TRANSPORTER, DOPAMINE (DAT)	0
204410	TRANSPORTER, NOREPINEPHRINE	-6
274030	TRANSPORTER, SEROTONIN (SERT)	-5

Figure 3 - Effect of RDX on [35S]TBPS Binding



8. DISCUSSION.

a. Contrary to earlier reports of increased salivation and lacrimation associated with RDX intoxication (Burdette et al., 1988; Crouse et al., 2006; Schneider et al., 1977), none of the RDX-treated rats in the current study showed evidence of increased secretions from the mouth or eyes in agreement with recent studies by Bannon (2010). The current observations do not support the hypothesis that RDX is a peripheral AChE inhibitor.

b. Analysis of the AChE activity in brains of RDX-treated rats collected at the time of seizure indicated that RDX had no effect of AChE activity and thus is not an inhibitor of brain AChE.

c. The correlation of blood RDX concentration with brain RDX concentration and time to seizure onset was poor; although suggestive, the correlation coefficient was only 0.5. This is most certainly related to pharmacokinetic issues related to the inhomogeneity of the RDX particle size in the dosing suspension and animal variability in compound absorption.

d. All animals that seized within the 30 min time frame mandated by the CHPPM IACUC had brain levels of RDX above 8 μg/g wet wt. This may represent the threshold level of RDX required for seizure initiation. In a 90 day toxicology study of RDX (Crouse et al., 2006), animals dosed daily with low doses of RDX, e.g. the LOAEL of 8 mg/kg/day developed seizures over the course of the experiment. The brain concentration of RDX in these seizing animals was not measured. We speculate that sequential low doses of RDX results in a slow accumulation of RDX in brain that reaches a threshold level of 8 µg/g wet wt., resulting in seizure onset.

e. In the current study only 6 out of 10 rats seized within 30 min. when dosed with RDX at 75 mg/kg with a suspension of 37.5 mg/kg, 2 ml/kg. This is in contrast to the 100% seizures induced with a similar dose, 75 mg/kg found within 15 min in a previous study (Way and McCain, 2007). They dosed with a suspension of 15 mg/ml, 5 ml/kg. The greater reliability and quickness of seizure onset in this prior study may be related to a more dilute suspension of compound administered and a faster absorption by the animal.

f. RDX binds to the convulsant site on the $GABA_A$ receptor with a K_i of 22 µM, an affinity similar to pentylenetetrazol, a convulsant commonly used in animal models of seizure and epilepsy (Coulter et al., 1990). RDX has a molecular weight of 222.3 daltons. Presuming a gram of brain equals 1 ml (although the extracellular space of brain is negligible), 8 µg/g wet wt. equals 36 µM. In a study using juvenile swine, animals seized at a brain concentration of 15 µM (Bannon, 2010). This suggests that sufficient RDX accumulates in brain to inhibit the majority of $GABA_A$ receptors in the brain, certainly sufficient to initiate seizure (Bigler, 1977; Ramakrishnan et al., 2005).

9. CONCLUSIONS.

a. RDX has significant affinity for the convulsant of the $GABA_A$ receptor. Thus, the mechanism of RDX-induced seizure is very likely to be dis-inhibition of excitatory neurons due to blockage of the inhibitory Cl^- current mediated by GABA (Bigler, 1977; Ramakrishnan et al., 2005).

b. AChE inhibition is not involved in the mechanism of seizure induction.

c. The concentration of RDX in brain must reach a level greater or equal to 8 µg/g wet wt. before seizure can begin. Higher brain concentrations can shorten the time to seizure onset.

d. These data will provide valuable information to the physiologically-based pharmacokinetic modeling being used to extrapolate rat data to human (Krishnan et al., 2009) and assist in reducing the interspecies uncertainty associated with extrapolating safe levels in animals with those for humans.

APPENDIX A

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APPENDIX B
QUALITY ASSURANCE STATEMENT

For: DTOX Study No. 87-XE-97HR9W-07, Protocol No 0BT9-60-09-05-01, titled "Mechanism of RDX-Induced Seizures in Rats", The following critical phases were audited by the Quality Systems Office:

Critical Phase Inspected/Audited (QSO Checklist #)	Date Inspected /Audited	Date Reported to Management
Protocol Review (QSO checklist # 1.2)	02/23/09	02/23/09
Test System - Receipt - Records Review– (#3.2)	09/11/09	09/16/09
Final Study Report Review (# 13.1)	09/11/09	09/16/09
Study Raw Data Review (#14.1)	09/11/09	09/16/09
Analytical Chemistry Support - Data Review (#16.1)	09/11/09	09/16/09

Note: All findings were made known to the Study Director at the time of the audit/inspection.

Michael P. Kefauver
GLP Assessor, Quality Systems Office

09/16/09
Date

Gene Sinar
Team Leader, Quality Systems Office

9/16/09
Date

APPENDIX C
ARCHIVES AND STUDY PERSONNEL

1. ARCHIVES

a. All raw data, documentation, records, protocol, and a copy of the final report generated as a result of this study will be archived in the storage facilities of the Toxicology Directorate, USACHPPM, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

b. Records on animal receipt, diet, and facility environmental parameters will be archived by the Veterinary Medical Division, Toxicology Directorate, for a minimum of five (5) years following submission of the final report to the Sponsor. If the report is used to support a regulatory action, it shall, along with all supporting data, be retained indefinitely.

c. The present study used the laboratory project number: . 87-XE-0BT9-09, protocol number 0BT9-60-09-05-01 for all filings.

d. The protocol, raw data, summary data, and the final report pertaining to this study will be physically maintained within Building E-2100, USACHPPM. These data may be scanned to a computer disk. Scanned study files will be stored electronically in Room 3010, Building E-2100, USACHPPM, APG, MD, 21010.

e. Archived SOPs may be found in Room 1026, Building E-2100, USACHPPM, APG, MD, 21010.

f. Records on animal receipt, diet, and environmental parameters are maintained in Room 3014, Building E-2100, USACHPPM, APG, MD, 21010.

g. Archivist: Kristin Newkirk.

2. PERSONNEL.

a. Manangement: Glenn Leach, Program Manager, Toxicity Evaluation Program (TEP)

b. Study Director: Dr. Larry Williams, Biologist, HERP.

c. Veterinary Support, Necropsies, and Animal Care: Anne MacLarty, DVM, MAJ.

d. Animal Care: Robert Sunderland, Theresa Hanna, Rebecca Kilby, TEP

3. ACKNOWLEDGEMENTS - We thank Mike Hable, DLS for running the RDX analysis in a timely and accurate manner.